Sequential expression and cooperative interaction of c-Ha-*ras* and c-*erbB* genes in *in vivo* chemical carcinogenesis

(7,12-dimethylbenzanthracene/hamster buccal pouch epithelium/stage-specific expression of cellular protooncogenes)

Zaheed Husain*, Yuanbio Fei*, Sayon Roy*, Dennis B. Solt†, Peter J. Polverini†, and Debajit K. Biswas*‡

*Laboratory of Molecular Biology, Harvard School of Dental Medicine, Boston, MA 02115; and †Department of Pathology, Northwestern University Medical School, Chicago, IL

ABSTRACT   The level of expression of several cellular protooncogenes is examined at different stages of 7,12-dimethylbenzanthracene (DMBA)-induced tumor development in hamster buccal pouch epithelium (HBPE). Results presented demonstrate overexpression of c-Ha-*ras* gene at a very early stage of tumor development, and this elevated level of expression of the gene persists throughout the tumorigenesis process. The expression of the cellular protooncogene c-*erbB*, on the other hand, can be detected only after 8–10 weeks of DMBA treatment of the tissue and increases with the progression of the disease. The overexpression of c-*erbB* gene can be correlated with the stage of extensive proliferation and subsequent invasion of the HBPE cells into the underlying connective tissue. This sequential pattern of stage-specific expression of the two cellular protooncogenes can be observed in (*i*) treated tissues, (*ii*) stage-representative cultured cells, and (*iii*) NIH 3T3 transformants derived with DNA from HBPE cells. The low-level expression of c-*myc* and c-*sis* genes detected in control tissues remains unaffected, while c-*fos* gene activity cannot be detected at any stage of tumor development. The overexpression of c-Ha-*ras* gene alone in HBPE cells derived from tissues treated for 5 weeks (DM5) is not sufficient to induce tumors in athymic mice, whereas expression of c-Ha-*ras* and c-*erbB* genes at later stages of tumor development (DM10 and HCPC cells) induce histopathologically defined epithelial cell carcinoma in athymic mice within 2–3 weeks. The sequential overexpression of c-Ha-*ras* and c-*erbB* genes in a stage-specific manner and their cooperative interaction in the DMBA-induced *in vivo* oral carcinogenesis have been demonstrated.

Carcinogenesis is believed to be a multistep process involving aberrant expression of a number of cellular inactive DNA sequences, the active forms of which are referred to as oncogenes (1, 2). The molecular mechanisms of chemical carcinogenesis have been studied by monitoring the accompanying altered structures and expressions (increases, decreases, inactivation, and unusual expressions) of these cellular protooncogenes. With *in vitro* DNA-mediated transformation studies, several investigators have proposed cooperative interaction of more than one cellular protooncogene, such as *ras* and *myc*, in the carcinogenesis process (3–10). The activation and overexpression of the *ras* family of genes in chemically induced benign growths, or growths that ultimately self-regress (11, 12), suggest a possible role for these genes in the chemical carcinogenesis process. It is proposed that activation of the *ras* gene may generate the signal necessary for the subsequent activation of cell proliferation-associated protooncogenes with the latter genes being involved in the progression of the *ras*-initiated cells to

malignancy (11). Besides the systems such as 7,12-dimethylbenzanthracene (DMBA)-induced skin carcinoma (13) and rabbit keratoacanthomas (12) in which aberrant expression of cellular protooncogenes are studied, there are very few *in vivo* carcinogenesis systems in which stepwise molecular analysis of the transformation process is conducted from the very early to the terminal stages of tumor development. In the present investigation, we have examined the molecular events in the *in vivo* carcinogenesis process in a well-defined animal model system (14) from the very beginning of DMBA treatment of the pouch tissue to the onset of the dysplastic and hyperplastic activity. The emphasis has been on the identification of early and stage-specific altered molecular events, such as activation of cellular protooncogenes, and to establish their role in this *in vivo* chemical carcinogenesis process. These molecular analyses are not restricted to the heterogenous population of cells in the tissue, but are also performed in the homogenous population of cultured hamster buccal pouch epithelium (HBPE) cells derived from the tissue at different stages of DMBA treatment (15) and also in NIH 3T3 transformants. The characteristic properties of transformed cells such as their (*i*) capability for passage under standard tissue culture conditions, (*ii*) anchorage-independent growth phenotype, (*iii*) capability to transform NIH 3T3 cells, and (*iv*) tumorigenic potential in athymic mice have been verified. In an *in vivo* chemical carcinogenesis system, we have demonstrated that the increased expression of the *ras* gene can be correlated with the initial transformation activity of the HBPE cells, whereas activation of the c-*erbB* gene can be correlated with the extensive proliferative and malignant phenotype of these cells in the intact animal. The sequential activation of the two cellular protooncogenes and the cooperative interaction of both the *ras* and *erbB* gene in the DMBA-induced *in vivo* oral carcinogenesis process is demonstrated.

MATERIALS AND METHODS

Male Syrian hamsters (70–80 g) were obtained from the Charles River Breeding Laboratories. One of the buccal pouch tissues was treated with 0.5% DMBA (Sigma) three times a week as described (14). Mineral oil was applied to the contralateral pouch, which was used as a control tissue for the subsequent experiments. Animals were sacrificed after specified periods of DMBA treatment by CO_2 inhalation. The control and treated pouch tissues were removed and washed with Hanks' buffered saline several times. A small portion was saved for histological examination, and the rest of the tissue was frozen in liquid N_2 and stored at $-80°C$ for nucleic acid isolation.

Abbreviations: DMBA, 7,12-dimethylbenzanthracene; HBPE, hamster buccal pouch epithelium.
‡To whom reprint requests should be addressed.

Cell Biology: Husain *et al.*

Proc. Natl. Acad. Sci. USA 86 (1989)   1265

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from the frozen tissue by the guanidinium isothiocyanate method (16) and subjected to Northern blot analysis under denaturing conditions (14, 16). The insert DNA from different recombinant plasmids was ^{32}P-labeled by nick-translation (16) and used as probe. The recombinant plasmids pAE-*Bam*HI with the *erbB* locus of the avian erythroblastosis virus (17), pc-fos-1 (human; ref. 18), and pv*Sis* (19) were obtained from American Type Culture Collection. The recombinant plasmid with MYC cDNA insert (human) was a gift from Philip Leder (Department of Human Genetics, Harvard Medical School). The c-Ha-*ras* plasmid (mouse cDNA) was from E. R. Scolnik of Merck Laboratories. The insert DNA from all the recombinant plasmids was used as a probe. The histone *H3.2* gene was originally cloned by Sittman *et al.* (20) and was provided by Arthur B. Pardee (Dana–Farber Cancer Institute). α_2-Tubulin cDNA clone was a gift from Joan Ruderman (21).

High molecular weight genomic DNA was isolated according to Gross-Bellard *et al.* (22) and subjected to Southern blot analysis (23). Establishment of cultured cells from control (PO) and treated tissue (DM3, DM5, and DM10) is described by Polverini and Solt (24). The HCPC cell line was derived from a DMBA-induced HBPE tumor (25). The epithelial nature of the cultured cells was characterized by electron microscopic examination and by immunochemical staining for high and low molecular weight cytokeratins (24).

The transformation of NIH 3T3 cells with DNA isolated from cultured PO, DM3, DM5, DM10, and HCPC cells was done according to the modified method of Wigler *et al.* (26) and Pasion *et al.* (27). Selection of foci and characterization of the transformants have been described (15). PO cells and DM3 cells did not induce transformation of NIH 3T3 cells nor could surviving foci be isolated. On the other hand, DM5, DM10, and HCPC DNA transformed NIH 3T3 cells with a frequency of 0.17, 0.36, and 0.47 foci per μg of donor DNA, respectively (15). The RNA and DNA isolated from the transformants of DM5 (DM5TX), DM10 (DM10TX), and HCPC (HCPCTX) cells were subsequently analyzed for the expression and structural organization of the cellular protooncogenes by Northern (16) and Southern (23) blot techniques. The anchorage-independent phenotypes of the transformants were examined by growth on soft agar.

Immunoprecipitation of the *ras* Gene p21 Protein Product. Metabolic labeling of cellular proteins in cultured HBPE cells with [^{35}S]methionine, preparation of the cell extract, and immunoprecipitation of p21 protein with monoclonal antibody Y13–259 were done (according to the method specified by the supplier of the monoclonal antibody (Oncogene Science, Manhasset, NY; ref. 28). The immunoprecipitated p21 protein was then analyzed by SDS/PAGE analysis (29). The electrophoretic mobility of immunoprecipitated p21 was compared to that observed in NIH 3T3 cells. The size of the immunoprecipitated protein was extrapolated from the mobility of the marker protein electrophoresed under identical conditions.

Tumor Induction in Athymic Mice. Athymic *nu nu* mice were obtained from the Frederick Cancer Center, National Cancer Institute, Frederick, MD. Approximately 1–2 $\times$ 10^6 cells of specified types were injected once subcutaneously. Control animals received the same number of 3T3 cells. Animals were examined periodically for the detection of palpable tumor formation.

RESULTS

Expression of Cellular Protooncogenes at Different Stages of DMBA-Induced HBPE Tumor Development. The level of expression of several cellular protooncogenes has been examined at different stages of tumor development in (*i*)

control (mineral oil-treated) and treated tissue; (*ii*) cultured cells derived from the control tissue (PO cells) and tissue after DMBA treatment for 3 weeks (DM3), 5 weeks (DM5), 10 weeks (DM10), and from a typical end stage tumor (HCPC); and (*iii*) NIH 3T3 transformants obtained after transfection with this stage-representative cultured cell DNA. The expression of cellular protooncogenes was measured by Northern blot analysis of cellular total RNA followed by hybridization with ^{32}P-labeled specific oncogene probe. A low level expression of the c-*sis* and c-*myc* genes can be detected and this level of expression of these two genes stays unaffected during the DMBA-induced transformation process. The c-*fos*-specific mRNA sequences cannot be detected at any stage of DMBA treatment or in cheek pouch tissue, cultured cells, or NIH 3T3 transformants. The expression levels of c-Ha-*ras* and c-*erbB* genes in treated HBPE cells, on the other hand, are altered during the DMBA-induced carcinogenesis process in a stage-specific manner.

The low level of expression of c-Ha-*ras* gene observed in control tissue was stimulated in HBPE cells treated for 5 weeks. This overexpression of the c-Ha-*ras* gene persists throughout the carcinogenesis process. The level of c-Ha-*ras*-specific mRNA sequences (1.4 kilobases, ^{32}P-labeled c-Ha-*ras*-hybridizable sequences) is significantly higher in 5-week treated tissue (Fig. 1 *Top*, lane 5) in HBPE cells established from 5-week treated tissue (DM5, Fig. 1 *Middle*) and also in NIH 3T3 transformants derived with DM5 DNA (DM5TX, Fig. 1 *Bottom*). The c-Ha-*ras* specific mRNA sequences are very low in control tissue (PO cells), in 3-week treated tissue (*Top*), and in DM3 (*Middle*), suggesting that the stimulation of this gene occurred some time between 3 and 5 weeks of DMBA treatment of HBPE cells. In parallel with the increased level of *ras*-specific mRNA (Fig. 1), the level of p21 protein is also elevated in DM5, DM10, and HCPC cells in comparison to that observed in 3T3 cells and DM3 cells (Fig. 1 *Inset*). The mobility of immunoprecipitated p21 protein is slightly decreased in *ras* overexpressing DM5, DM10, and HCPC cells in comparison with that observed in 3T3 and DM3 cells. Altered mobility of p21 protein has also been observed in many *ras* overexpressing tumor cells and has been identified with a specific mutation within the gene leading to single amino acid substitution in the peptide chain (30). The decreased mobility of immunoprecipitated p21 protein observed in *ras* overexpressing DM5, DM10, and HCPC cells may be due to such a mutation in the *ras* gene of DMBA-treated HBPE cells.

The expression of the c-*erbB* gene shows a completely different pattern during the DMBA-induced carcinogenesis process in HBPE cells. Two species of c-*erbB*-specific mRNA sequences can be detected in HPBE cells, the predominant one being the 1.5-kilobase species. The c-*erbB*-specific mRNA sequences can be detected only after 8–9 weeks of DMBA treatment of the tissues, and this level continues to increase with the progression of the carcinogenesis process (Fig. 2 *Top*). Similarly, c-*erbB*-specific mRNA can be detected only in DM10 cells, HCPC cells (Fig. 2 *Middle*), and DM10TX and HCPCTX cells (Fig. 2 *Bottom*). No c-*erbB*-specific mRNA sequences can be detected in treated tissues at an earlier stage, in cultured cells (i.e., PO, DM3, and DM5), or in NIH 3T3 transformant DM5TX cells. The c-*erbB* gene activation seems to be an event that follows activation of the c-Ha-*ras* gene, occurring at an earlier stage during the DMBA-induced transformation of HBPE cells. A sequential pattern in the activation of these two cellular protooncogenes is demonstrated in this *in vivo* carcinogenesis system.

We have examined the expression level of both c-Ha-*ras* and c-*erbB* genes in 3 of 20 NIH 3T3 transfectants obtained with DM10 and HCPC DNA and observed overexpression of both genes in all three. In such transfections, cotransfer of
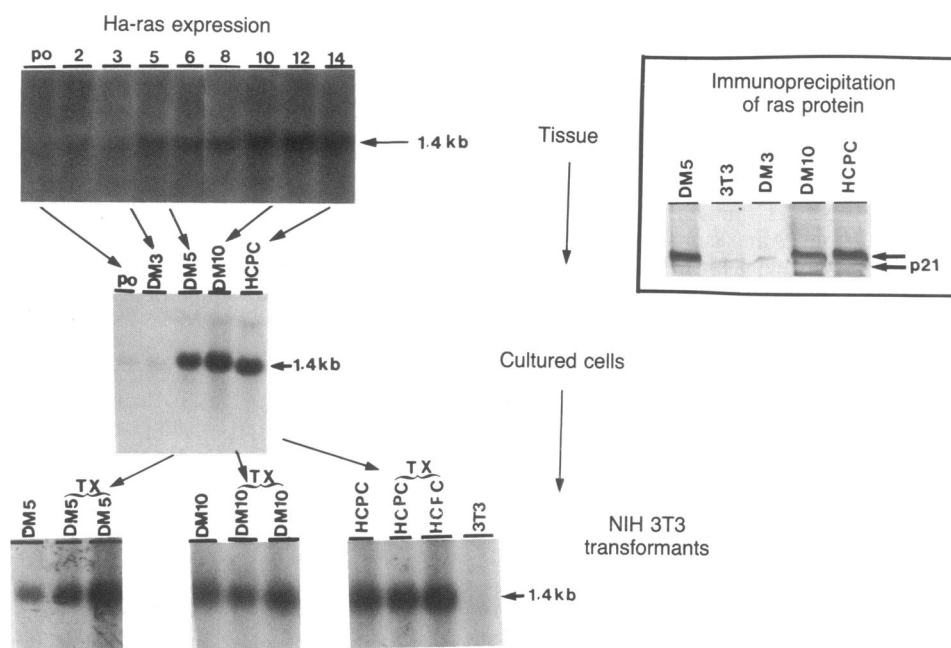
FIG. 1.   Level of expression of c-Ha-*ras* gene in DMBA-treated HBPE tissue in stage-representative cultured cells and in transformants. The level of expression of the c-Ha-*ras* gene in DMBA-treated hamster cheek pouch tissue (*Top*), in stage-representative cultured HBPE cells (15) (*Middle*), and in NIH 3T3 transformants is determined by Northern blot analysis of the cellular total RNA (16). Total cellular RNA (50 μg) from control (PO) and treated tissue (number of weeks of DMBA treatment shown above each lane) are applied (*Top*). Cellular total RNA (25 μg) from cultured HBPE cells (*Middle*) and from NIH 3T3 transformants derived with DNA from DM5 (DM5TX), DM10 (DM10TX), and HCPC (HCPCTX) cells (*Bottom*) are analyzed similarly. The ^{32}P-labeled c-Ha-*ras* insert DNA is used as a probe. Two transformants of each cell type are examined. The numbers indicated by the arrows on the right side of the panels show the size of c-Ha-*ras*-specific mRNA sequence. (*Inset*) The level of p21 protein in cultured HBPE cells as identified by immunoprecipitation with *ras* antibody (monoclonal, Y13–259), followed by SDS/PAGE analysis (28). An aliquot of cell extracts of each strain with equivalent radioactivity (cpm) has been used for immunoprecipitation. Arrow shows the mobility of the p21 protein as determined from the size (M_r, 21,000) of this autoradiographic band calculated from the mobility of several marker proteins (BRL). kb, Kilobases.

two genes located in two different chromosomes in the single transformed focus of the recipient cells is a rare event. It is, however, not ruled out that the above is a circumstantial event in the small number of transfectants analyzed. Other possible mechanisms of this observed phenomenon are discussed.
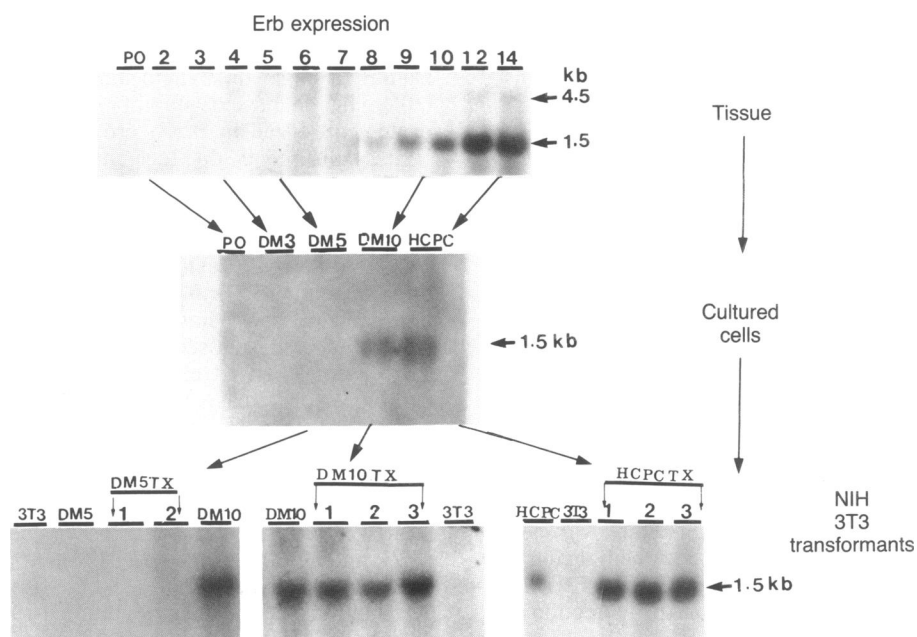

FIG. 2.   Level of expression of c-*erbB* gene in tissues, stage-representative cultured HBPE cells, and NIH 3T3 transformants. The level of expression of c-*erb* gene in control (PO) and treated tissue (number of weeks of DMBA treatment indicated by number above each lane; *Top*), cultured HBPE cells (*Middle*), and NIH 3T3 transformants (*Bottom*) is determined by Northern blot analysis of the cellular total RNA (as described in Fig. 1). RNA (50 μg) isolated from control and treated tissue (*Top*) and RNA (25 μg) from cultured cells (*Middle*) and NIH 3T3 transformants were applied in each lane. The *erbB* cDNA insert was ^{32}P-labeled and used as a probe. Two DM5TX cells, three DM10TX cells, and four HCPCTX cells were analyzed. The numbers with arrows indicate the size (kilobases) of the *erbB*-hybridizable-specific mRNA sequences.
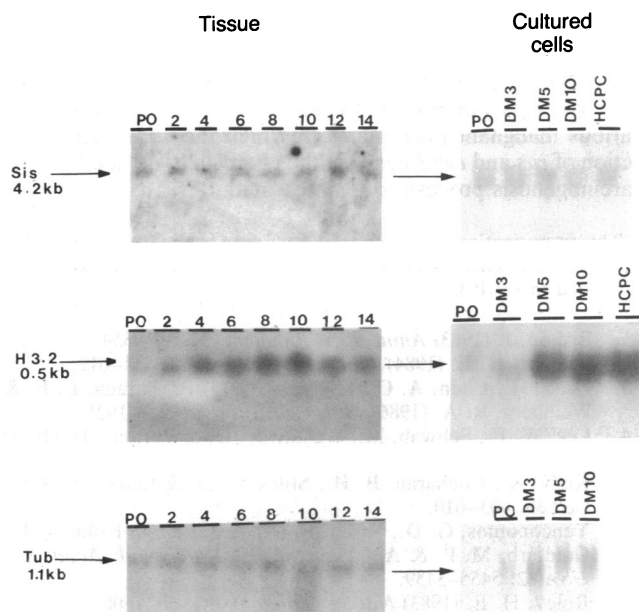
Cell Biology: Husain *et al.*

Proc. Natl. Acad. Sci. USA 86 (1989)   1267

Tissue   Cultured cells

FIG. 3. Level of expression of c-*sis*, H3.2, and α_2-tubulin gene in HBPE tissue and cultured cells. Northern blot analysis of the cellular total RNA from control and DMBA-treated tissue (*Left*, number above each lane indicates weeks of treatment) and from cultured HBPE cells are carried out as described above. RNA (50 μg) from the tissue and RNA (25 μg) from the cultured cells were applied in each lane as described in Fig. 1. cDNA insert sequences for v-*sis* (American Type Culture Collection), α_2-tubulin (21), and whole plasmid H3.2 (20) were ^{32}P-labeled and used as probes. Numbers with arrows indicate the size of the specific mRNA species (kilobases).

Simultaneous analysis of other cellular genes, such as tubulin and histone H3.2, demonstrates that the expression of the cell-cycle-dependent H3.2 gene expression can be correlated with the proliferative and nonproliferative stages of HBPE cells (Fig. 3 *Middle*), whereas the level of expression of

the α_2-tubulin gene remains the same throughout the carcinogenesis process (Fig. 3 *Bottom*). This pattern of expression of H3.2 and α_2-tubulin is maintained both in treated tissues and in cultured cells derived from the tissue (Fig. 3).

Tumorigenic Potential of DM5, DM10, and HCPC Cells. The tumorigenic potential of cultured cells derived from the treated tissues at different stages of DMBA-induced tumor development has been verified by transplantation into athymic mice. Tumor formation in athymic mice by *ras*-expressing DM5 cells is very poor in comparison to that observed with the *ras* and *erbB*-expressing DM10 and HCPC cells. Upon transplantation of DM10 and HCPC cells under the back skin (once, 10^6 cells per animal) of athymic mice, the palpable and visible growth was induced within 2 weeks, showing accelerated tumorous growth within 6–8 weeks (Fig. 4 *Right*). Transplantation of the same number of 3T3 or DM5 cells (Fig. 4 *Left*) do not induce any visible growth within 8 weeks in athymic mice. However, this does not eliminate the possibility that DM5 cells may induce tumors in athymic mice after a long lag period. All three transformation phenotypes— i.e., anchorage-independent growth, transformation of NIH 3T3 cells (15), and tumor induction in athymic mice—are exhibited at a comparatively much reduced level in DM5 cells than those observed with DM10 and HCPC cells. With the same level of *ras* gene expression as DM10 and HCPC cells, DM5 cells do not demonstrate the same potency of tumor formation in athymic mice or other phenotypes of fully transformed cells. Subsequent activation of one more cellular protooncogene—i.e., the *erbB* gene—seems to be necessary to acquire the property of fully transformed cells or at least for the acceleration of the process.

DISCUSSION

The role of concurrent overexpression of two of these cellular protooncogenes—e.g., c-Ha-*ras* and c-*myc* gene—in the transformation process has been implicated by several investigators with DNA-mediated transfection studies in *in*
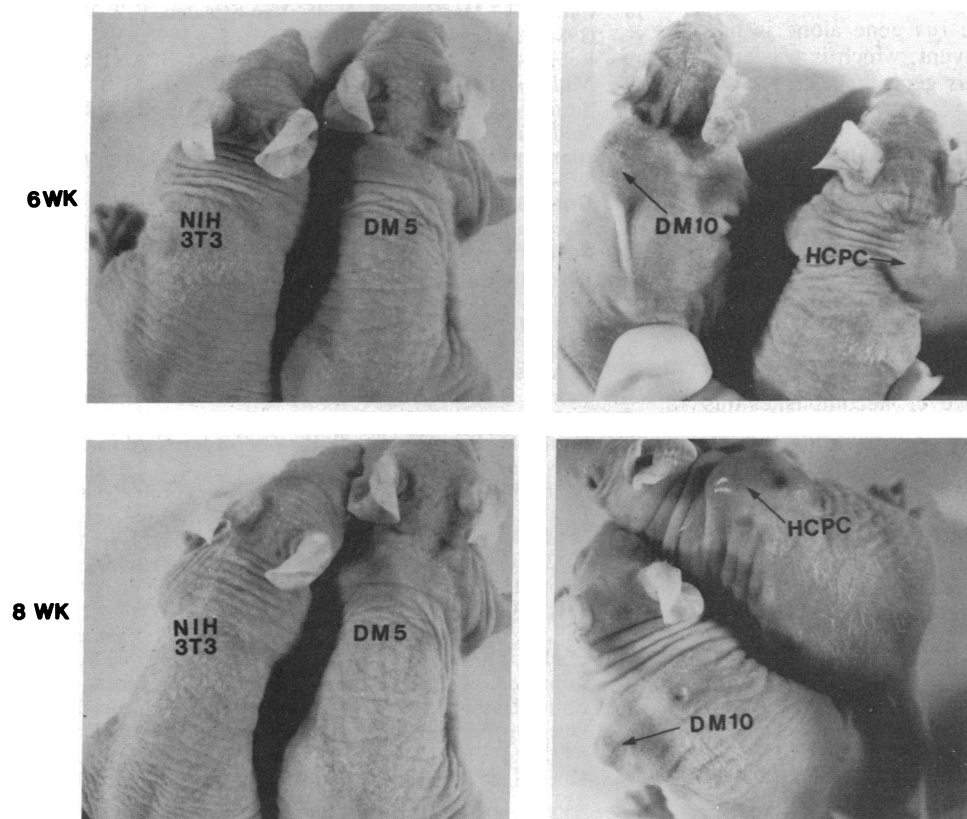

FIG. 4. Tumor induction in athymic mice by DM5, DM10, and HCPC cells. Cells (10^6) from each of these stage-representative cell types were transplanted under the back skin of athymic mice (in triplicate) (*nu nu* mice from the Frederick Cancer Center) under sterile conditions. The athymic mice were housed separately from other animals under controlled room temperature. In control animals, the same number of NIH 3T3 cells were transplanted. Palpable tumor formation was examined weekly. All three animals show similar results (a typical example is shown here). (*Upper*) Tumor induction after 6 weeks. (*Lower*) Same as in *Upper*, examined and photographed 8 weeks after transplantation of the cells. In the case of DM10 and HCPC cells, tumorous growths are visible after 2 weeks. Histological examination of the tumors establishes that these growths are epithelial cell carcinoma.

vitro cultured cell systems (3–10). Infection of mouse skin with Harvey murine sarcoma virus (HaMSV) did not show neoplastic activity until the skin surface was treated with tumor promoter phorbol 12-myristate 13-acetate (PMA) (13). The HaMSV and PMA-induced skin papillomas and carcinomas both demonstrated activation of the *ras* gene; however, only a small percentage of papillomas progressed to carcinomas. These results suggest that the *ras* gene product alone is not sufficient for the full manifestation of the malignant phenotype. Subsequent molecular events, such as overproduction of transforming growth factor type β, have been implicated in the carcinogenesis system (31). The overexpression of the *ras* gene seems to be an early event involved in the initiation process, which then triggers the signal for the subsequent events required for the progression of the carcinogenesis process. It seems that the role of other cellular protooncogenes (probably the ones related to cell proliferation) is to drive the *ras*-initiated cells to the malignant state.

Papillomas and carcinomas represent histopathologically altered tissues and thus do not represent precancerous or very early stages of tumorigenesis. The DMBA-treated HBPE represents an *in vivo* model, development of which is close to human oral epidermoid carcinoma. The molecular analysis of the tumor development has been carried out in stages in which no significant histopathological changes can be detected and thus reflects the molecular changes in the true precancerous stages of *in vivo* tumor development. In this system, the carcinogenesis process progresses histopathologically as well. Furthermore, the molecular changes observed in the heterogenous population of cells in the treated tissues are identical to those observed in the homogenous population of cultured epithelial cells derived from these tissues. Continuous treatment of the pouch tissue for 5 weeks, or of cells derived from such short-term treated tissue (DM5), demonstrate certain transformation phenotypes; however, the frequency and intensity of the properties of DM5 cells are dissimilar to those observed with latter (DM10) or end stage (HCPC) tumor cells (15). These results strongly suggest that overexpression of the *ras* gene alone is not sufficient, whereas the additional event, which is triggered after the initial activation of the *ras* gene, accelerates the malignant transformation process. Activation of the c-*erbB* gene during week 9–10 of DMBA treatment, in addition to earlier activation of the *ras* gene in HBPE cells, may have provided sufficient signals to drive these cells to the fully transformed state. The cooperativity of the action of both activated *ras* and *erbB* genes is evident in the HBPE cells treated with DMBA for 10 weeks or more. Although activation of the *ras* gene alone in HBPE cells (DM5) shows a certain transformation phenotype at a very low level, it is not sufficient to induce malignant growth in athymic mice. The activated *ras* gene in cooperation with the activated *erbB* gene in DM10 and HCPC cells, however, accomplishes this within a very short period (2–3 weeks) following transplantation of these cells in athymic mice.

The mechanism of the observed overexpression of both c-Ha-*ras* and c-*erbB* genes in the same NIH 3T3 transfectants is not yet clear. Possible translocation of the amplified c-*erbB* gene commonly observed in many tumor cells is not ruled out (32). Alternatively, the observed overexpression of the c-*erbB* gene may be a posttransfectional event induced by activated c-Ha-*ras* gene in the recipient NIH 3T3 cells. Nevertheless, the latter possibility seems remote because c-*erbB* gene expression cannot be detected in the transfectants established with DM5 cell DNA, which carries the activated *ras* locus. Influence of a combination of many different factors on the complex tumorigenic process in Chinese hamster embryo fibroblasts has also been postulated by Ober and Pardee (33,

34). It may also be possible that after a long latency DM5 cells with only elevated *ras* may even induce tumors in athymic mice. All these results, however, do not completely rule out their roles as potential individual factors in the genesis of various malignant phenotypes in which the cooperativity of action of *ras* and *erbB* genes in the chemically induced *in vivo* carcinogenesis process is demonstrated.

1. Bishop, J. (1983) *Annu. Rev. Biochem.* **52**, 301–354.
2. Vermus, H. E. (1984) *Annu. Rev. Genet.* **18**, 553–612.
3. Land, H., Chen, A. C., Morgenstern, J. P., Parada, L. F. & Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6**, 1917–1925.
4. Lee, W. F., Schwab, M., Westaway, D. & Vermus, H. (1985) *Mol. Cell. Biol.* **5**, 3345–3346.
5. Kelly, K., Cocharan, B. H., Stiles, C. D. & Leder, P. (1983) *Cell* **35**, 603–610.
6. Yancopoulos, G. D., Nisen, P. D., Tesfaye, A., Kohl, N. E., Goldfarb, M. P. & Alt, F. W. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5455–5459.
7. Ruley, H. E. (1983) *Nature (London)* **304**, 602–606.
8. Balmain, A. & Pregnell, I. B. (1983) *Nature (London)* **303**, 72–74.
9. Quintanilla, M., Brown, K., Ramsden, M. & Balmain, A. (1986) *Nature (London)* **322**, 78–80.
10. Bizub, D., Wood, A. W. & Skalka, A. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6048–6052.
11. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56**, 779–827.
12. Leon, J., Kamino, H., Stenberg, J. J. & Pellicer, A. (1988) *Mol. Cell. Biol.* **8**, 786–793.
13. Brown, K., Quintanilla, M., Ramsden, M., Derr, F. B., Young, S. & Balmain, A. (1986) *Cell* **46**, 447–456.
14. Wong, D. T. W. & Biswas, D. K. (1987) *Oncogene* **2**, 67–72.
15. Solt, D. B., Polverini, P. J., Ray, S., Fei, Y. & Biswas, D. K. (1988) *Carcinogenesis,* in press.
16. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
17. Venstrom, B. & Bishop, J. M. (1982) *Cell* **28**, 135–143.
18. Curren, T., MacConnell, N. P., Van-Straaten, F. & Verma, I. (1984) *Mol. Cell. Biol.* **3**, 914–941.
19. Robbins, K. C., Devare, S. K. & Aaronson, S. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2918–2922.
20. Sittman, D. B., Gravers, R. A. & Marzluff, W. F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1849–1853.
21. Alexzandraki, D. & Ruderman, J. V. (1983) *J. Mol. Evol.* **19**, 397–410.
22. Gross-Bellard, M., Oudet, P. & Chambon, P. (1973) *Eur. J. Biochem.* **36**, 32–38.
23. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
24. Polverini, P. J. & Solt, D. B. (1986) *Lab. Invest.* **54**, 432–441.
25. Odukoya, O., Schwartz, J., Weichselbaum, R. & Shklar, G. (1983) *J. Natl. Cancer. Inst.* **71**, 1253–1263.
26. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. & Axel, R. (1977) *Cell* **11**, 223–230.
27. Pasion, S. G., Hartigan, J. A., Kumar, V. & Biswas, D. K. (1987) *DNA* **6**, 419–427.
28. Furth, M. E., Aldrich, T. H. & Cordon-Cardo, C. (1987) *Oncogene* **1**, 47–58.
29. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–682.
30. Fasano, O., Aldrich, T., Fuyuniko, T., Taparowsky, E., Furth, M. & Wigler, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4008–4012.
31. Akhurst, R. J., Fee, F. & Balmain, A. (1988) *Nature (London)* **331**, 363–365.
32. Gill, G. N., Bertics, P. J., Thompson, D. M., Weber, W. & Cochet, C. (1985) in *Cancer Cells: Growth Factors and Transformation,* eds. Feramisco, T., Ozane, B. & Stiles, C. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 11–18.
33. Ober, S. S. & Pardee, A. B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2766–2770.
34. Ober, S. S. & Pardee, A. B. (1987) *J. Cell. Physiol.* **132**, 311–317.